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# Replication of Herpes Simplex Virus Type 1 on Hydroxyurea-Resistant Baby Hamster Kidney Cells

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Hydroxyurea-resistant ($HU^r$) baby hamster kidney cells were isolated, subcloned, and characterized. One clonal line, which contained elevated levels of ribonucleotide reductase, lost its HU resistance during passage in the absence of the inhibitor, whereas another clonal line was stably resistant. The replication of herpes simplex virus type 1 on these cells was compared with that of the parvovirus minute virus of mice. Herpes simplex virus type 1 was found to be as sensitive to HU on both lines of $HU^r$ baby hamster kidney cells as it was on parental (HU-sensitive) cells, whereas parvovirus replication was about eight times more resistant on $HU^r$ baby hamster kidney cells compared with the parental cells. The results suggest that herpes simplex virus type 1 cannot use the cellular reductase and may code for its own.

The composition and genetic origin of ribonucleotide reductase activity in herpes simplex virus (HSV)-infected cells is not known. The enzyme carries out the reduction of ribonucleoside diphosphates to their deoxy forms (rNDP → dNDP), the levels of which in turn regulate activity and which in all systems examined are comprised of two nonidentical subunits (for a review, see reference 20). In mammalian cells, the M1 subunit binds the nucleoside triphosphate effectors, and the M2 subunit is responsible for the sensitivity of the enzyme to the ribonucleotide reductase-specific inhibitor hydroxyurea (HU) (1, 5, 19). Two properties of the enzyme present in HSV-infected cells, in which dTTP levels are elevated (3, 9), differentiate it from the reductase in the uninfected cells: (i) resistance to feedback inhibition by dTTP and (ii) reduced requirements for $Mg^{2+}$ and ATP (8, 11, 17). This finding suggests that the M1 subunit may be virus coded or virus modified. For the herpesvirus pseudorabies virus, it has been shown directly that reductase activity in infected cells is unaffected by antibody specific for the mammalian M1 subunit (12). That equine herpes virus replication is naturally resistant to HU (4) suggests that this virus may code for its own M2 subunit. However, replication of HSV is sensitive to HU (16, 21), as is the infected cell enzyme in vitro (11). The possibility therefore exists that this part of the HSV-induced reductase activity is contributed by the host cell.

As an approach to examining whether HSV can utilize the cellular ribonucleotide reductase or is dependent upon a virus-induced or virus-modified enzyme, we have studied the replication of HSV on $HU^r$ baby hamster kidney (BHK) cells. As a control, we have compared this replication with that of the parvovirus minute virus of mice (MVM), which relies largely upon cellular enzymes for its replication and is unlikely to code for its own reductase (for a review, see reference 22).

Although chinese hamster ovary (CHO) cells which are $HU^r$ have been established (13), CHO cells are nonpermissive for HSV replication (7). We therefore isolated $HU^r$ BHK cells by single-step selection in medium containing 0.8 mM HU. The properties of two clonal lines of $HU^r$ BHK cells, 1A-0.8 and 2A-0.8, are shown in Table 1. The cell lines grew equally well with or without 0.8 mM HU added to the medium and had longer generation times than the parental BHK line. Clone 1A-0.8 (maintained in 0.8 mM HU) reproducibly expressed elevated levels of $HU^s$ ribonucleotide reductase activity over a period of months in culture. After ca. 30 cell doublings in medium without HU (1A-0), these cells reverted to the normal BHK phenotype with lower ribonucleotide reductase levels, shorter generation times, and inability to tolerate 0.8 mM HU in the medium. Clone 2A-0.8 (maintained in 0.8 mM HU) exhibited a less marked elevation in ribonucleotide reductase activity, which was more resistant to HU in vitro. During identical prolonged culture without

TABLE 1. Properties of BHK and HU$^r$ BHK cells

| Cells[a] | Generation time (h)[b] | | Sp act of ribonucleotide reductase[c] | |
|---|---|---|---|---|
| | −HU | +HU | −HU | +HU |
| BHK | 16 | Cell death | 0.79 | 0.48 |
| 1A-0.8 | 22 | 26 | 2.28 | 0.80 |
| 1A-0 | 17 | Cell death | 0.78 | 0.49 |
| 2A-0.8 | 24 | 26 | 1.73 | 0.70 |
| 2A-0 | 23 | 25 | 1.42 | 0.85 |

[a] BHK-21 clone 13 cells (15) were grown in 10% calf serum (Colorado Serum Co.), 10% tryptose phosphate (Difco), and Dulbecco modified Eagle medium and transferred 1:10 every 3 to 4 days as described previously (6). HU$^r$ BHK cells were established as follows: $10^6$ cells per dish were seeded, and fluid was changed in medium containing 0.8 mM HU every 4 days. After 6 weeks, four colonies had grown which were pooled and transferred as a population of uncloned HU$^r$ BHK cells. At passage 2, these cells were cloned twice. Four stable clones were obtained, two of which (1A-0.8 and 2A-0.8) were used for this study between passage levels 3 and 13 after subcloning. 1A-0 and 2A-0 represent the same cell lines grown in the absence of HU for 10 passages (ca. 30 cell doublings).

[b] Cell cultures were seeded at a dilution factor of 1:10 and counted in duplicate with a Coulter Counter daily for 5 consecutive days. Generation times were determined graphically from the exponential part of the respective growth curves.

[c] Subconfluent monolayers of cells were washed twice with Tris-buffered saline, scraped from the dish, and frozen as dry pellets at −90°C. Extracts were prepared by sonication of the thawed cell pellets in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffer (HEPES, pH 7.2) and 1 mM dithiothreitol. After removal of the debris by centrifugation, ribonucleotide reductase activity was measured in the supernatant as follows: the standard reaction mixture (100 μl) contained 20 mM HEPES (pH 7.2), 0.06 mM $FeCl_3$, 2.7 mM magnesium acetate, 8.3 mM NaF, 6.2 mM dithiothreitol 5mM ATP, 0.05 mM CDP, 2.5 μCi of [5-$^3$H]CDP (10 to 30 Ci/mmol), and 800 μg of protein (measured by the method of Bradford [2]). HU was added at a concentration of 1 mM where indicated. After incubation at 37°C for 30 min (within the linear phase of enzyme activity), the reaction was stopped by the addition of perchloric acid to a final concentration of 1 M. The diphosphates were converted to their monophosphate form and separated as described by Huzar and Bacchetti (8), except that chromatography was for 18 h instead of 40 h. Specific activity is expressed as nanomoles of dCMP formed per hour per milligram of protein.

HU (2A-0), this clone and its enzyme did not revert to the normal BHK phenotype and remained stably resistant to HU. Thus, these two HU$^r$ BHK clones are clearly different. The resistance of 1A-0.8 is most likely mediated by some unstable form of gene amplification, a phenomenon already established for both HU$^r$ and methotrexate-resistant CHO cells (10, 14) and for HU$^r$ mouse 3T6 cells (1). Other types of HU$^r$ CHO cells have been shown to contain mutations in the genes involved (14), and this is a more likely mechanism for the stably resistant phenotype expressed by clone 2A-0.8.

We then examined the ability of each cell type to support replication of HSV-1 and MVM in the absence of HU (Table 2). Although HSV-1 production was 10-fold less when grown on HU$^r$ BHK cells, this was not an effect of slower growth rates but represented the final yields of which these cells were capable (data not shown). MVM, on the other hand, grew to higher titers on HU$^r$ BHK compared with parental BHK cells for reasons not understood. The effect of HU upon virus replication was studied by assaying the final yields of virus produced in medium containing four different concentrations of HU. From the resulting inhibition curves, the amount of HU required to inhibit the replication of each virus by 50% ($ID_{50}$) was determined (Table 2). For the parvovirus MVM grown on sensitive BHK cells, the $ID_{50}$ was ca. eightfold less (0.13 mM) than that of MVM when grown on HU$^r$ BHK cells (0.81 mM on 1A-0.8 and 1.12 mM on 2A-0.8). MVM therefore, demonstrated an HU$^s$ phenotype on sensitive cells and assumed a resistant phenotype on HU$^r$ cells. In contrast, the $ID_{50}$ for HSV type 1 (HSV-1) did not vary with cell phenotype, and the virus was uniformally sensitive to HU ($ID_{50}$ = 0.25 to 0.31 mM HU). Similar observations were made for HSV-2 (data not shown). HSV-2 was inherently less sensitive to HU than was HSV-1 even on parental BHK cells, but no change in its HU sensitivity was observed when grown on HU$^r$ BHK cells.

The biochemical properties of the ribonucleotide reductase present in HSV-infected cells provide strong evidence that an M1-type subunit comprises a part of that enzyme activity and that it is virus coded or virus modified (8, 11). The data presented here demonstrate that HSV, in contrast to MVM, retains its HU$^s$ phenotype on HU$^r$ BHK cells, irrespective of the mechanism by which these cells have become resistant. This behavior would most easily be explained if HSV does code for and depends on its own M2 subunit. We are currently attempting to isolate an HU$^r$ HSV-1 mutant by using HU$^r$ BHK cells; this would provide definitive proof of a viral origin for the enzyme.

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TABLE 2. Sensitivity of HSV-1 and MVM replication to HU on BHK and HU$^r$ BHK cells

| Virus | Cells | | | | | |
|---|---|---|---|---|---|---|
| | BHK | | 1A-0.8 | | 2A-0.8 | |
| | Virus yield (PFU/cell)[a] | $ID_{50}$ (mM HU)[b] | Virus yield (PFU/cell) | $ID_{50}$ (mM HU) | Virus yield (PFU/cell) | $ID_{50}$ (mM/HU) |
| HSV-1[c] | $2.3 \times 10^3$ | 0.31 | $2.3 \times 10^2$ | 0.25 | $2.0 \times 10^2$ | 0.25 |
| MVM[d] | $7.5 \times 10^2$ | 0.13 | $1.45 \times 10^3$ | 0.81 | $1.5 \times 10^3$ | 1.12 |

[a] Duplicate samples of cells were infected with either virus at a mutiplicity of 5 PFU per cell. After incubation at 37°C for 72 h (a time chosen because cytopathic effect was complete and virus yields were maximal), cells were harvested into the medium and sonicated (HSV-1) or frozen and thawed three times (MVM), and total virus was determined by plaque assay.

[b] Duplicate samples of cells were infected (see footnote *a*), and media containing four different HU concentrations (1 to 4 mM) were added after adsorption. Incubation was at 37°C for 72 h, virus yields were then determined by plaque assay. These were plotted against the HU concentration, and the $ID_{50}$s were determined graphically.

[c] HSV-1 Glasgow strain 17 was grown and assayed by plaque formation as described previously (6).

[d] MVM strain p was grown and assayed by plaque formation as described previously (18; B. A. Spalholz, J. Bratton, D. C. Ward, and P. Tattersall, *in* E. M. Scolnick and A. J. Levine, ed., *Cetus-UCLA Symposium. Tumorviruses and Differentiation*, in press).

## LITERATURE CITED


1. **Akerblom, L., A. Ehrenberg, A. Gräslund, H. Lankinen, P. Reichard, and L. Thelander.** 1981. Overproduction of the free radical of ribonucleotide reductase in hydroxyurea-resistant mouse fibroblast 3T6 cells. Proc. Natl. Acad. Sci. U.S.A. **78**:2159–2163.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Cohen, G. H.** 1972. Ribonucleotide reductase activity of synchronized KB cells infected with herpes simplex virus. J. Virol. **9**:408–418.
4. **Cohen, J. C., M. L. Perdue, C. C. Randall, and D. J. O'Callaghan.** 1975. Replication of equine herpes virus type 1: resistance to hydroxyurea. Virology **67**:56–67.
5. **Engström, Y., S. Eriksson, L. Thelander, and M. Akerman.** 1979. Ribonucleotide reductase from calf thymus. Purification and properties. Biochemistry **18**:2941–2948.
6. **Francke, B.** 1977. Cell-free synthesis of herpes simplex virus DNA: conditions for optimal synthesis. Biochemistry **16**:5655–5664.
7. **Francke, U., and B. Francke.** 1981. Requirement of the human chromosome 11 long arm for replication of herpes simplex virus type 1 in non-permissive chinese hamster X human diploid fibroblast hybrids. Somat. Cell Genet. **7**:171–191.
8. **Huszar, D., and S. Bacchetti.** 1981. Partial purification and characterization of the ribonucleotide reductase induced by herpes simplex virus infection of mammalian cells. J. Virol. **37**:580–588.
9. **Jamieson, A. T., and G. Bjursell.** 1976. Deoxyribonucleotide triphosphate pools in herpes simplex type 1 infected cells. J. Gen. Virol. **31**:101–113.
10. **Kaufman, R. J., and R. T. Schimke.** 1981. Amplification and loss of dihydrofolate reductase genes in a chinese hamster ovary cell line. Mol. Cell. Biol. **1**:1069–1076.
11. **Langelier, Y., and G. Buttin.** 1981. Characterization of ribonucleotide reductase induction in BHK-21/C13 syrian hamster cell line upon infection by herpes simplex virus (HSV). J. Gen. Virol. **57**:21–31.
12. **Lankinen, H., A. Gräslund, and L. Thelander.** 1982. Induction of a new ribonucleotide reductase after infection of mouse L cells with pseudorabies virus. J. Virol. **41**:893–900.
13. **Lewis, W. H., and J. A. Wright.** 1978. Genetic characterization of hydroxyurea-resistance in chinese hamster ovary cells. J. Cell. Physiol. **97**:73–86.
14. **Lewis, W. H., and J. A. Wright.** 1978. Ribonucleotide reductase from wild-type and hydroxyurea resistant chinese hamster ovary cells. J. Cell. Physiol. **97**:87–98.
15. **MacPherson, I. A., and M. G. P. Stoker.** 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**:147–151.
16. **Nil, S., S. Rosenkranz, C. Morgan, and H. M. Rose.** 1968. Electron microscopy of herpes simplex virus. III. Effect of hydroxyurea. J. Virol. **2**:1163–1171.
17. **Ponce de Leon, M., R. J. Eisenberg, and G. H. Cohen.** 1977. Ribonucleotide reductase from herpes simplex (type 1 and 2) infected and uninfected KB cells: properties of the partially purified enzymes. J. Gen. Virol. **36**:163–173.
18. **Tattersall, P.** 1972. Replication of parvovirus MVM. I. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. **10**:586–590.
19. **Thelander, L., S. Eriksson, and M. Akerman.** 1980. Ribonucleotide reductase from calf thymus. Separation of the enzyme into two non-identical subunits, proteins M1 and M2. J. Biol. Chem. **255**:7426–7432.
20. **Thelander, L., and P. Reichard.** 1979. Reduction of ribonucleotides. Ann. Rev. Biochem. **48**:133–158.
21. **Wagner, E., R. Swanstrom, and M. Stafford.** 1972. Transcription of the herpes simplex virus genome in human cells. J. Virol. **10**:675–682.
22. **Ward, D. C., and P. Tattersall.** 1978. Replication of mammalian parvoviruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.